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(54) Title: METHOD OF SUPPRESSING TUMOR FORMATION IN VIVO BY ADMINISTERING JE/MLP-1 (57) Abstract A method of suppressing tumor formation in a vertebrate by administering JE/MCP-1 is described. Also described are methods of treating localized complications of malignancies and methods of combatting parasitic infection by administering JE/MCP-1.		

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-1-

METHOD OF SUPPRESSING TUMOR FORMATION IN VIVO BY ADMINISTERING JE/MLP-1

Background

Cancer results when a vertebrate's own cells become malignant. Healthy individuals at any given time carry
5 potentially malignant cells in their body. These cells are generally recognized and killed by the individuals' immune system. However, some malignant cells are not destroyed by the immune system and proliferate into tumors.

10 Currently, there are not adequate and specific therapies for cancer. For example, surgical excision of tumors is not an effective method of treatment where the cancer has metastasized. In addition, radiation and chemotherapy often kill normal cells in addition to
15 cancerous cells.

Another problem is that chemotherapeutic agents follow first-order kinetics. As a result, a constant percentage, rather than a constant number of cells are killed by a given application of a chemotherapeutic agent.
20 Consequently, malignant cells, which could cause a relapse in the disease, remain even when a patient is diagnosed as having complete clinical remission.

A method of suppressing cancer that employs the individual's own immune system would be useful.

25 Summary of the Invention

The present invention relates to Applicant's finding that expression of the JE/MCP-1 protein in malignant cells suppresses their ability to form tumors in vivo. Thus,

-2-

the invention comprises, in one embodiment, a method of suppressing tumor formation in a vertebrate by administering to the vertebrate a therapeutically effective amount of JE/MCP-1. The protein can be administered alone
5 or as an adjuvant to surgery or cytotoxic chemotherapy.

The suppressive effect of JE/MCP-1 depends on the induction of the vertebrate's immune response, specifically the response of monocytes. Thus, in another embodiment, the invention comprises a method of increasing a
10 vertebrate's monocyte-mediated tumoricidal activity in vivo by administering to the vertebrate an effective amount of JE/MCP-1.

JE/MCP-1 can also be administered to treat localized complications of malignancy. For example, JE/MCP-1 could
15 be used to inhibit malignant pleural effusions or ascites. Therefore, in a further embodiment, the invention comprises methods of inhibiting pleural effusion or ascites in a vertebrate by locally administering JE/MCP-1 to the anatomic spaces between the lung and the pleural membrane
20 or the stomach and the peritoneum.

In a further embodiment tumor killing cells, such as tumor infiltrating lymphocytes (TIL cells) are genetically engineered to express the JE/MCP-1 protein. The engineered cells therefore can be administered to a vertebrate to
25 provide a synergistic local tumor cell killing.

The presence of JE/MCP-1 in vivo is accompanied by an local increase in the presence of eosinophils. Therefore, another aspect of the subject invention comprises methods of combatting a parasitic infection in a vertebrate animal
30 by administering to that vertebrate an effective amount of JE/MCP-1.

-3-

A major advantage of using JE/MCP-1 in treating cancer is that it employs the individual's own immune system and therefore would have fewer side-effects than conventional chemotherapies. In addition, JE/MCP-1 stimulates monocytes and, as such, does not depend on a total immunologic response.

Brief Description of the Drawings

Figure 1 is an autoradiograph of polyacrylamide gel electrophoresis of cells of DUKX-B11 cells transfected with pXM alone (OA-2, OB-2, and OA-10), pXM-JE1 (1A-2 and 1B-2), pXM-JE10 (10A-2, 10B-2, and 10A-10), or pXM-hJE34 (hJEC-10 and hJEC-100).

Figure 2 is a graph plotting the growth rate of tumors. Each symbol represents a different mouse. A. CHO cells only, 10^7 OA-2 cells (open square); 10^7 OA-2 cells plus 10^7 hJEC-10 cells (closed square and closed circle); 10^7 OA-2 cells and 10^7 10A-10 cells (closed triangle and open triangle); (identical results were obtained with 10^7 OA-2 cells and 10^7 hJEC-100 cells). B. CHO and HeLa cells. 10^5 HeLa cells and 10^7 OA-2 cells (open square and open circle); 10^5 HeLa cells and 10^7 hJEC-10 cells (closed square and closed circle); 10^5 HeLa cells and 10^7 10A-10 cells (closed triangle and open triangle); (identical results were obtained with 10^7 HeLa cells and 10^7 hJEC-100 cells in 3 out of 4 animals).

Figure 3 is a photograph (magnification 400X) of hematoxylin and eosin-stained cellular infiltrate elicited by A. OA-10, B. 10A-10 or C. hJEC-10 cells.

-4-

Figure 4 is a photograph (magnification 800X) of hematoxylin and eosin-stained cellular infiltrate elicited by hJEC-10 cells.

Detailed Description of the Invention

- 5 The present invention is based on the finding that expression of the JE/MCP-1 protein in malignant cells suppresses their ability to form tumors in vivo.
- The JE gene is a platelet-derived growth factor (PDGF)-inducible "competence" or "early response gene" first identified in mouse 3T3 cells (Cochran, B.H. Cell 10 33:939-947 (1983)). Sequence and expression analysis showed that unlike other early response genes, such as c-myc, c-fos, or c-jun, the murine JE gene encodes a secreted glycoprotein with cytokine-like properties
- 15 (Kawahara, R.S. J. Biol. Chem 264:679-682 (1989); Rollins, B.J. Proc. Natl. Acad. Sci. USA 85:3738-3742 (1988)). The human homolog of murine JE has been cloned, (Rollins, B.J. Mol. Cell. Biol. 9:4687-4695 (1989)), and the predicted amino acid sequence of its protein is identical to that of
- 20 a monocyte chemoattractant, MCP-1 (Yoshimura, T., J. Exp. Med. 169:1449-1459 (1989) (Yoshimura T. JE. FEBS Lett. 244:487-493 (1989)) also called MCAF (Furutani, Y. et al., Biochem. Biophys. Res. Commun. 159:249-255 (1989); Matsushima, K. et al., J. Exp. Med. 169:1485-1490 (1989) and SMC-CF (Graves, D.T., et al., Science 245:1490-1493 (1989); Valente, A.J. et al. Biochem 27:4162-4168 (1988)).

 The JE/MCP-1 protein is structurally related to the members of a large, recently identified family of low molecular weight secreted proteins that appear to be

-5-

involved in the inflammatory response (Leonard, E.J. and T. Yoshimura Immunol. Today 11:97-101 (1990); Rollins, B.J. et al., Mol. Cell. Biol. 9:4687-4695 (1989); Wolpe, S.D. and A. Cerami, FASEB J. 3:2563-2573 (1989)). The
5 genes for many of these proteins, including human JE/MCP-1, are clustered on chromosome 17q11.2-12 (Donlon, T.A. et al., Genomics 6:548-553 (1990); Irving, S.G., et al., Nucleic Acids Res. 18:3261-3270 (1990); Rollins, B.J., et al., Genomics (in press)) or mouse chromosome 11
10 (Wilson, S.D., et al., J. Exp. Med. 171:1301-1314 (1990)). These genes are also related to the genes encoding another family of cytokines, whose members include the neutrophil activator NAP-1/IL-8 (Peveri, P., et al., J. Exp. Med. 167:1547-1559 (1988); Schroder, J.M. et al. J. Immunol
15 139:3474-3483 (1987); Yoshimura, T. et al., Proc. Natl. Acad. Sci. USA 84:9233-9237 (1987)), many of which cluster at 4q12-21 (Griffin, C.A. et al., Cytogenet Cell Genet 45:67-69 (1987); Luster, A.D. et al. Proc. Natl. Acad. Sci. USA 84:2868-2871 (1987); Richmond, A. EMBO J. 7:2025-
20 2033 (1988)).

JE/MCP-1 exerts several effects specifically on monocytes. Both natural and recombinant JE/MCP-1 are potent chemoattractants for human monocytes in vitro, (Matsushima, K. et al. J. Exp. Med. 169:1485-1490 (1989);
25 Yoshimura, T. et al. J. Exp. Med. 169:1449-1459 (1989)) and purified recombinant JE/MCP-1 can stimulate an increase in cytosolic free calcium and the respiratory burst in monocytes (Zachariae, C.O.C., et al., J. Exp. Med. 171:2177-2182 (1990); Rollins, B.J. et al. Blood (in
30 press)). Purified natural JE/MCP-1 has also been reported

-6-

to activate monocyte-mediated inhibition of tumor cell growth, but not tumor cell killing, in vitro (Matsushima, K. et al. J. Exp. Med. 169:1485-1490 (1989)).

The following demonstrates that expression of the JE
5 gene in malignant cells suppresses their ability to form tumors in vivo. This apparent phenotypic reversion requires interaction with host factors in vivo, since expression of JE/MCP-1 does not alter the transformed character of these cells in vitro. Furthermore, the
10 following shows that JE/MCP-1-expressing cells exert their effect in trans by their ability to suppress tumor formation when co-injected with JE/MCP-1-non-expressing tumor cells.

In order to create malignant cells expressing
15 JE/MCP-1, the DHFR deletion mutant CHO cell line, DUKXB-11 (G. Urlaub and L.A Chasin, Proc. Natl. Acad. Sci. USA, 77:4216-4220 (1980)) was transfected with the expression vector pXM (Yang, Y.-C. et al. Cell 47:3-10 (1986) containing a variety of JE cDNA species. High levels of
20 JE/MCP-1 protein expression were achieved in stably transfected lines by methotrexate (MTX)-induced DNA amplification. R.J. Kaufman, et al., EMBO J. 6:187-193 (1987).

Figure 1 shows JE/MCP-1 protein expression in in-
25 dependently derived cell lines selected for resistance to 2 or 10 uM MXT. There is no detectable JE/MCP-1 protein secreted from cell lines transfected with pXM alone (cell lines OA-2 and OB-2; cell line OA-10 was derived from OA-2) or with pXM containing murine JE cDNA in the anti-
30 sense orientation (1A-2 and 1B-2). Considerable JE/MCP-1

-7-

prot in was secreted by c ll lines transfected with murine JE cDNA in the sense orientation (10A-2 and 10B-2; 10A-10 was derived from 10A-2) and human JE cDNA (hJEC-10). Cell line hJEC-100, derived from hJEC-10 by selecting for
5 resistance to 100 uM MTX, also secreted human JE/MCP-1 protein.

Murine JE cDNA directs CHO cells to secrete a micro-heterogeneous protein of M_r 27-39,000, similar to the natural protein and the protein expressed in a COS cell
10 expression system. Rollins, B.J. et al., Proc. Natl. Acad. Sci. USA 85:3738:3742 (1988)). Nearly half of the apparent M_r is due to O-linked glycosylation. Human JE/MCP-1 proteins expressed in CHO cells are also similar to native and COS cell expressed JE/MCP-1 α (M_r 15,000) and
15 JE/MCP 1 β (M_r 11,000). (Rollins, B.J., et al. Mol. Cell. Biol. 9:4687-4695 (1989))

The monocyte chemoattractant activities (MCA) secreted by these cell lines were determined as described in detail in Example 1. They were 1415 U/24hr/ 10^6 cells
20 (10A-2), 1079 U/24hr/ 10^6 cells (10B-2), 3008 U/24hr/ 10^6 cells (10A-10), 54 U/24hr/ 10^6 cells (hJEC-10), and 692 U/24hr/ 10^6 cells (hJEC-100) JE/MCP-1-non-expressing cells secreted 10-30 U/24hr/ 10^6 cells. The increased MCA secreted by murine JE/MCP-1 expressing lines is due to
25 increased JE/MCP-1 protein in the medium, not to higher specific activity. Murine JE/MCP-1 appears to be more stable than human JE/MCP-1 in culture, perhaps due to its more extensive glycosylation. (Rollins, B.J., et al. Mol. Cell. Biol. 9:4687-4695 (1989)).

-8-

By several criteria, JE/MCP-1 expression did not alter the transformed phenotype of CHO cells in vitro. Table I shows that while doubling times of each of the independently derived cell lines varied considerably, the average doubling time of all the JE/MCP-1-expressing lines (25.1 hrs \pm 5.7 sd) was nearly identical to the average doubling time of the JE/MCP-1-non-expressors (26.4 hrs \pm 5.7 sd). Also, JE/MCP-1 expression did not alter the transformed cellular morphology of the CHO cells.

10 Finally, all cell lines formed colonies in soft agar.

- 9 -

TABLE 1. PROPERTIES OF TRANSFECTED CHINESE HAMSTER OVARY CELL LINES.

Cell Line	JE cDNA	Doubling		Soft Agar Colonies/25 mm ²	No. Cells Tumors/Animal	
		Time (hours)			Injected	Injected
<u>JE/MCP-1 Non-expressors</u>						
0A-2	None	24.2	91 ± 14.0	2 x 10 ⁷	1/1	
"	"	"	"	1 x 10 ⁷	2/2	
"	"	"	"	2 x 10 ⁶	3/4	
0A-10	None	20.4	32 ± 1.9	1 x 10 ⁷	2/2	
0B-2	None	19.0	73 ± 2.6	1 x 10 ⁷	0/4	
1A-2	Antisense	30.7	75 ± 14.5	8 x 10 ⁶	2/2	
1B-2	Antisense	31.2	74 ± 1.7	1 x 10 ⁷	1/2	
<u>JE/MCP-1 Expressors</u>						
10A-2	Murine	28.8	72 ± 7.3	2 x 10 ⁷	0/1	
"	"	"	"	1 x 10 ⁷	0/2	
"	"	"	"	2 x 10 ⁶	0/4	
10B-2	Murine	17.8	86 ± 5.1	1 x 10 ⁷	0/2	
10A-10	Murine	30.0	129 ± 6.8	1 x 10 ⁷	0/2	
hJEC-10	Human	28.8	32 ± 6.8	1 x 10 ⁷	0/2	

In vivo, however, JE/MCP-1 expression led to a striking difference in behavior. Table 1 shows that all but one (0B-2) of the JE/MCP-1-non-expressing cell lines formed large subcutaneous tumors that appeared within

-10-

three weeks of injection into nude mice. In contrast, all of the JE/MCP-1-expressing lines, including the human JE/MCP-1 expressing lines, formed no tumors for as long as ten months after injection. At autopsy, there was no
5 microscopic evidence of residual tumor in the animals that received JE/MCP-1-expressing cells.

These observations suggested the possibility that JE/MCP-1-secreting cells attracted monocytes to the site of tumor cell injection and once there, secreted JE/MCP-1
10 protein induced monocyte tumoricidal activity. To test this hypothesis, JE/MCP-1-expressing cells were mixed with 10^7 OA-2 cells, a number of cells that reproducibly led to tumor formation when injected alone (see Table 1).

Figure 2A shows that co-injection of murine JE/MCP-1-
15 expressing cells (10A-10) with OA-2 cells completely suppressed tumor formation in two animals. Co-injection of high-level human JE/MCP-1-expressing cells (hJEC-100) with OA-2 cells also completely suppressed tumor formation. Co-injection of low-level human JE/MCP-1-
20 expressing cells (hJEC-10) suppressed tumor formation for 8-10 days, after which tumors appeared. Presumably hJEC-10 cells exerted a suppressive effect transiently until the proliferating OA-2 cell mass reached a size that enabled it to escape the effect.

25 JE/MCP-1-expressors and non-expressors were also co-injected with HeLa cells to test whether JE/MCP-1 expression could suppress tumor formation by another cell type. Figure 2B shows that 10A-10 cells completely suppressed the formation of tumors by HeLa cells. As
30 above, co-injection with hJEC-10 cells delayed tumor

-11-

formation. In a separate experiment, 4 of 4 animals injected with 10^7 HeLa cells formed tumors, while only 1 of 4 animals injected with 10^7 HeLa cells plus 10^7 hJEC-100 cells formed tumors. Mice that displayed no tumor growth after receiving HeLa plus hJEC-100 cells were then injected with 10^7 HeLa cells alone. These mice developed large tumors within 14 days, indicating that prior suppression of HeLa cell tumor growth in the presence of JE/MCP-1 does not render mice immune to rechallenge with HeLa cells.

Histologic examination of the tumors arising from co-injected HeLa and OA-2 cells revealed a mixture of epithelioid HeLa cells and spindle-shaped CHO cells. Examination of the tumors formed in animals that received HeLa and hJEC-10 cells also demonstrated a mixture of HeLa cells and CHO cells. The presence of some hJEC-10 cells in these tumors was confirmed by Northern blot analysis in which expression of human JE mRNA could be detected. After reaching a certain size, these tumors may overwhelm the host response elicited by the low levels of human JE/MCP-1 secreted by hJEC-10 cells. However, tumor growth still requires the presence of a malignant JE/MCP-1-non-expressing cell line, since hJEC-10 cells injected by themselves cannot form tumors (Table 1). This suggests again that the intrinsic growth properties of the CHO cells have not been altered by JE/MCP-1 expression, and that the human JE/MCP-1 expressors will proliferate in vivo if they are protected by an enlarging mass of malignant cells.

Figures 3 and 4 show the results of a histological examination of the sites of CHO cell inoculation 24 hours

-12-

after cells were injected. JE/MCP-1-non-expressors (darkly stained, large cells indicated by arrows in Figure 3A) induce the appearance in the underlying connective tissue of only a few cells, most of which are neutrophils. In contrast, CHO cells expressing murine JE/MCP-1 (indicated by arrows in Figure 3B) elicited an abundant cellular infiltrate. At higher power (Figure 4A), it is apparent that this infiltrate consisted primarily of monocytes with a reproducibly significant proportion of eosinophils (arrows), usually 10-20%. As might be expected, low-level human JE/MCP-1-expressing cells (hJEC-10) induced a qualitatively similar infiltrate (Figure 4B) that was intermediate in intensity between the non-expressors and the murine JE/MCP-1 expressors (compare Figure 3C to Figures 3A and 3B).

The results represented by Figure 3 and 4 demonstrate a predominantly monocytic infiltrate at the site of tumor cell injection, suggesting that monocytes mediate tumor growth suppression. The effect is probably not mediated by T lymphocytes or by natural killer cells, since purified recombinant JE/MCP-1 has no stimulatory effect on natural killer cells in vitro.

If monocytes are responsible for tumor suppression, there are several possible mechanisms whereby JE/MCP-1-activated monocytes might exert their effects. JE/MCP-1 could induce the expression of a soluble mediator of tumor cells lysis such as tumor necrosis factor (TNF). L.J. Old, Science, 230: 630-632 (1985). J.L. Urban et al., Proc. Natl. Acad. Sci. USA 83: 5233-5237 (1986). Alternatively, TNF could be expressed and displayed in an active form on the cell surface of activated monocytes. M. Kriegler, et al., Cell 53: 45-52 (1988).

-13-

Inter stingly, an eosinophilic component to the inflammatory cell infiltrate was also observed. This may be either a direct effect of JE/MCP-1 or, JE/MCP-1 may induce the expression of another factor with eosinophil
5 chemoattractant properties.

These results point to a clinical role for infused JE/MCP-1 in vertebrate animals, such as humans. For example, JE/MCP-1 can be administered to patients with cancer. This would necessarily be limited to patients
10 with low tumor loads, i.e. as an adjuvant to surgery or cytotoxic chemotherapy. Systemically infused JE/MCP-1 would lead to a generalized increase in the activation state of a vertebrate's monocytes. There is some evidence that patients harboring malignancies have depressed
15 monocyte function. W.G. Chaney et al., Cell Molec. Genet., 5: 15-27 (1986). E.S. Kleinerman et al., Lancet ii: 1102-1105, (1980). If the defect were cytokine based, rather than an inherent monocyte defect, JE/MCP-1 infusion would correct the abnormality.

20 Alternatively, JE/MCP-1 may prove useful in treating localized complications of malignancy, such as pleural effusions or ascites. Instilling JE/MCP-1 into the involved anatomic space (e.g. the space between the lung and the pleural membrane or the space between the stomach
25 and the peritoneum) can lead to local monocyte accumulation and activation.

As a therapeutic, JE/MCP-1 can be administered to vertebrate animals (i.e. animals having an immune system), including humans. The compounds of this invention can be
30 employed in admixture with conventional excipients, i.e.,

-14-

pharmaceutically acceptable organic or inorganic carrier substances suitable for parental or topical application.

Suitable pharmaceutical carriers include, but are not limited to water, salt solutions, alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethyl-cellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., enzyme inhibitors, to further reduce metabolic degradation.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages.

For topical application, there are employed as nonsprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, (e.g.,

-15-

preservatives, stabilizers, wetting agents, buffers of salts for influencing osmotic pressure, etc.). For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., a Freon.

It will be appreciated that the actual preferred amounts of JE/MCP-1 in a specific case will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, and the particular situs and organism being treated. Dosages for a given host can be determined using conventional considerations, (e.g., by means of an appropriate, conventional pharmacological protocol.).

Alternatively, tumor killing cells, such as tumor infiltrating lymphocytes (TIL cells) could be genetically engineered to express the JE/MCP-1 protein. Tumor killing cells engineered in this way can provide synergistic local tumor cell killing. The tumor killing cells could be engineered in vitro and administered to the vertebrate or the tumor killing cells could be engineered in vivo into the vertebrate's own supply of tumor killing cells using methods which are known in the art.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

-16-

EXAMPLESExample 1 In Vivo Tumoricidal Effect of JE/MCP-1

Cell culture. DUKXB-11 cells (Urlaub, G. and L.A. Chasin, Proc. Natl. Acad. Sci. USA, 83:5233-5237 (1987))
5 (dihydrofolate reductase (DHFR) mutant Chinese hamster ovary (CHO) cells) were grown in the alpha modification of minimal essential medium (MEM- α) without ribonucleosides and deoxyribonucleosides, supplemented with 10% bovine calf serum and 10 ug/ml adenosine, deoxyadenosine, and
10 thymidine (MEM- α /BCS/ATT). (Kaufman, R.J. et. al. EMBO J. 6: 187-193, (1989)). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum and antibiotics.

Transfections and Methotrexate (MTX) selection.

15 DUKXB-11 cells were grown in 10 cm culture dishes as described above. At 60% confluence, medium was removed and replaced with fresh MEM- α /BCS/AAT supplemented with 3 ug/ml polybrene and incubated overnight. (Chaney, W.G. et al., Somat. Cell Molec. Genet., 12:237-244, (1986)). The
20 next day, each dish was given 3 ml fresh MEM- α /BCS/AAT with 83 ng/ml plasmid DNA and 10 ug/ml polybrene. Cells were incubated at 37°C for 6 hr with rocking every 90 min. Medium was then removed and replaced with 5 ml fresh MEM- α /BCS/AAT containing 30% DMSO for exactly 4 min.
25 Medium was removed, the cells were washed once with MEM- α , and given 10 ml of MEM- α /BCS/AA. Cells were incubated at 37°C for 48 hr, then trypsinized and re-plated into 4 culture dishes (10 cm) in nucleoside- and deoxynucleoside-free MEM- α supplemented with 10% dialyzed BCS, and

-17-

refed with this medium every 3 days. Two independent transfections were performed using pXM (Yang et al. Cell 47:3-10 (1986); pXM-JE10 (murine JE cDNA (Rollins, B.J., et al., Proc. Natl. Acad. Sci. USA 85:3738-3742 (1988)) in the sense orientation), pXM-JE1 (murine JE cDNA in the antisense orientation), and pXM-hJE34 (human JE cDNA (Rollins, B.J., et al., Mol. Cell. Biol. 9:4687-4695 (1989)). Colonies from each independent transfection that grew in ribonucleoside- and deoxyribonucleoside-free medium were trypsinized and combined. Step-wise selection in increasing concentrations of methotrexate (MTX) was carried out at the following levels: 0.02 uM, 0.1 uM, 0.5 uM, 2.0 uM, 10.0 uM, and 100.0 uM. At each concentration, surviving colonies were trypsinized and pooled.

Protein analysis. Confluent cell cultures were incubated in methionine-free MEM- α with 2% dialyzed BCS for 45 min, then changed to 0.5 ml of the same medium with 500 uCi [³⁵S]methionine (DuPont NEN, Boston, MA). Cells were incubated at 37°C for 4 hrs. after which the medium was collected, made 1 mM in phenylmethylsulfonyl fluoride (PMSF), centrifuged to remove cells and debris, and stored at -70°C. Immune precipitations using anti-JE/MCP-1 antiserum were performed (Rollins, B.J., et al., Mol. Cell. Biol. 9:4687-4695 (1989)) and the results analyzed by electrophoresis through an SDS-containing 17% polyacrylamide gel.

Soft agar colony formation assay. Five thousand cells were suspended in MEM- α containing 10% dialyzed BCS, 0.3% agar, and the appropriate concentration of MTX.

-18-

While still molten, this suspension was distributed on a gelled 4 ml underlayer of MEM- α containing 10% dialyzed BCS, 0.6% agar, and the appropriate concentration of MTX in a 60 mm culture dish. Cells were fed with 3 drops of fresh medium every 5 days. After 14 days, colonies consisting of greater than 50 cells were counted.

Monocyte chemoattractant activity (MCA). Confluent monolayers of CHO cells were incubated in serum-free MEM- α for 24 hr, after which the medium was centrifuged to remove cells and debris, and the remaining adherent cells were trypsinized and counted. Fresh human peripheral blood mononuclear cells were purified from the blood of volunteer donors by centrifugation on a cushion of Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Cells at the interface were washed twice in Gey's balanced salt solution (GBSS) with 2% BSA, then resuspended at 4×10^6 cells/ml in GBSS with 0.2% BSA, and MCA was measured in a 48-well microchamber apparatus. (Falk, W., et al., J. Immunol. Methods 33:239-247 (1980)). The concentration of MCA in CHO cell medium was defined as the reciprocal of the dilution showing half-maximal activity. (Yoshimura, T., et al., J. Exp. Med. 169:1449-1459 (1989)).

Nude mouse injections. Cells were suspended in 0.2 ml PBS and injected subcutaneously into 4-week old male Swiss nu/nu mice. Mice were monitored daily for tumor growth. Tumor volume was derived by multiplying the values of three perpendicular diameters.

-19-

Equivalents

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention
5 describe herein. Such equivalents are intended to be encompassed by the following claims.

-20-

CLAIMS

1. A method of suppressing tumor formation in a vertebrate animal comprising administering to said vertebrate a therapeutically effective amount of JE/MCP-1.
- 5 2. A method of increasing monocyte mediated tumoricidal activity in a vertebrate animal comprising administering to said vertebrate an effective amount of JE/MCP-1.
- 10 3. A method of treating a localized side-effect of malignancy in a vertebrate animal comprising locally administering to the vertebrate a therapeutically effective amount of JE/MCP-1.
- 15 4. The method of Claim 3 wherein the side effect is selected from the group consisting of pleural effusions or ascites.
5. A method of suppressing tumor formation in a vertebrate comprising administering to said vertebrate tumor killing cells which express JE/MCP-1.
- 20 6. A method of Claim 5, wherein the tumor killing cells are tumor infiltrating lymphocytes.
7. A method of combatting a parasitic infection in a vertebrate animal comprising administering to the vertebrate animal an effective amount of JE/MCP-1.

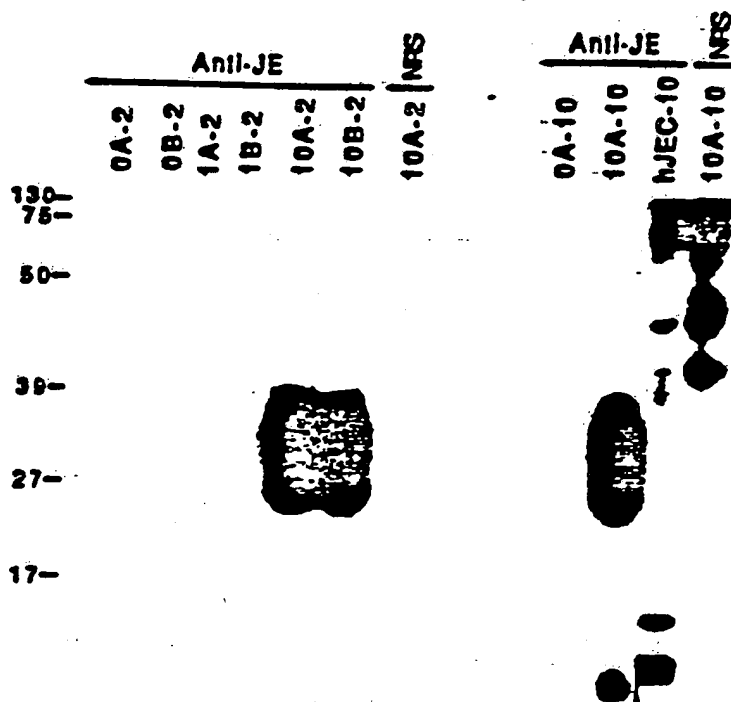


FIGURE 1

2/4

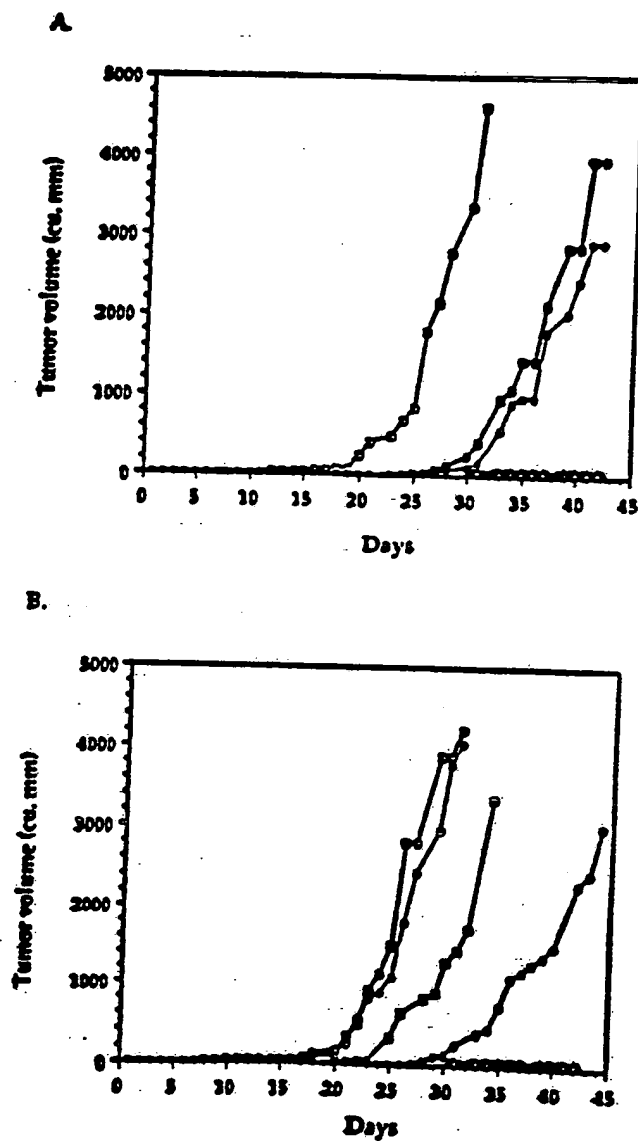


FIGURE 2

3/4

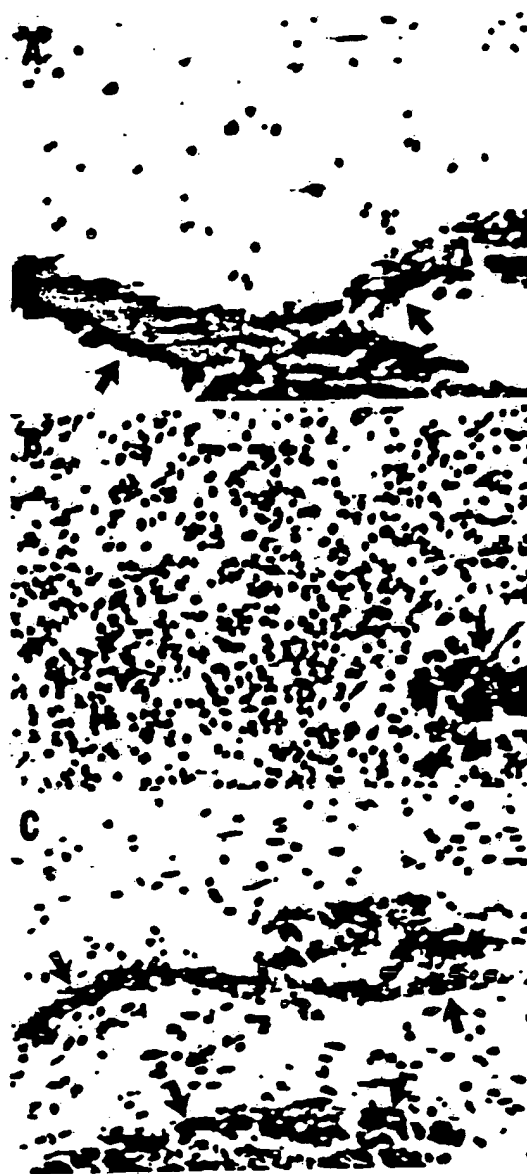


FIGURE 3

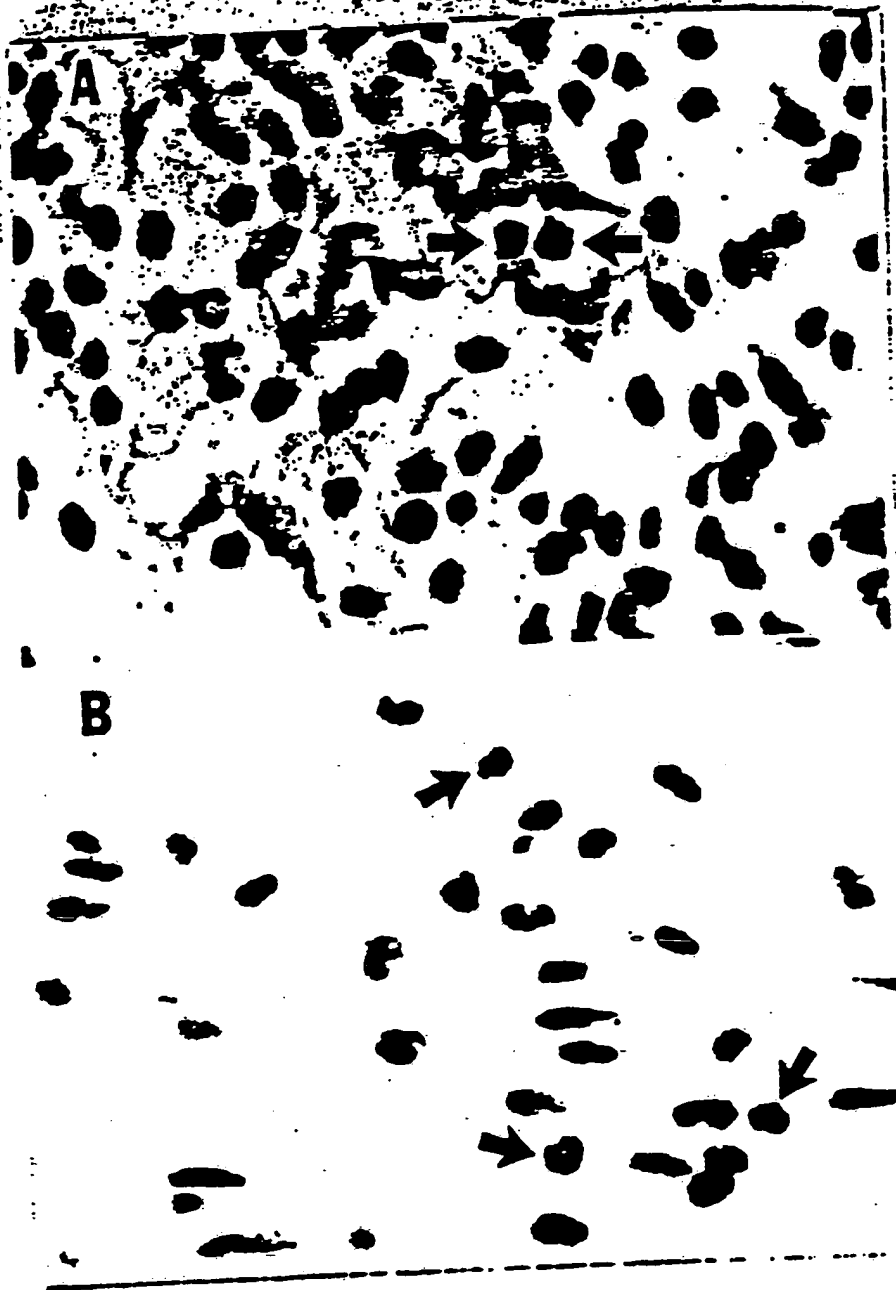


FIGURE 4

INTERNATIONAL SEARCH REPORT

PCT/US 92/04099

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K37/66; C07K15/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; A61K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	CYTOKINE vol. 1, no. 1, November 1989, pages 2 - 13; KOUJI MATSUSHIMA ET AL.: 'Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL1 and TNF' see page 10, left column, paragraph 2 - page 11, left column, paragraph 1	1-3
A	THE JOURNAL OF EXPERIMENTAL MEDICINE vol. 169, no. 4, 1 April 1989, pages 1485 - 1490; KOUJI MATSUSHIMA ET AL.: 'Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line' see page 1487, paragraph 2 - page 1489, paragraph 1	1,2
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
20 AUGUST 1992	01.09.92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B. <i>[Signature]</i>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/ 04099

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-7 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.